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# Heavy chain genes of rabbit IgG: Isolation of a cDNA encoding γ heavy chain and identification of two genomic $C_\gamma$ genes

(allotype/nucleotide sequence/recombinant phage DNA library)

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**ABSTRACT** **A cDNA library was constructed by using rabbit spleen poly(A)$^+$RNA as template, and from this library was isolated a cDNA clone, p2a2, that encodes 179 amino acids of the heavy chain of rabbit IgG. The nucleotide sequence of p2a2 showed that it encodes the COOH-terminal eight amino acids of the CH1 domain, the hinge region, the CH2 domain, and the $NH_2$-terminal half of the CH3 domain of $C_\gamma$. Southern blot hybridization analysis of rabbit sperm DNA showed that two *Eco*RI fragments hybridized strongly with the $C_\gamma$ cDNA. The p2a2 cDNA was used as a probe to isolate recombinant Charon 4A phage clones containing $C_\gamma$ sequences from a genomic library of rabbit liver DNA. Two distinct DNA segments were identified by restriction mapping and hybridization analysis, suggesting that the haploid rabbit genome may contain two different $C_\gamma$ genes.**

Genetic variants, or allotypes, of rabbit Ig heavy and light chains have been described and extensively studied (1–3). The allotypic specificities of the variable regions of heavy chains ($V_H$a, $V_H$x, and $V_H$y) and of the constant regions of the heavy chains of IgM ($C_\mu$n), IgG ($C_\gamma$de), and IgA ($C_\alpha$f and $C_\alpha$g) appear to be encoded by genes at several closely linked loci ($V_Ha$, $V_Hx$, $V_Hy$, $C_\mu n$, $C_\gamma de$, $C_\alpha f$, and $C_\alpha g$). At each locus, multiple alleles have been described that appear to be inherited in simple mendelian fashion. Recent studies, however, have shown that small amounts of Ig bearing allotypic determinants not predicted by mendelian inheritance patterns can be found in normal serum (4, 5) as well as on the surface of B lymphocytes (6, 7). For example, a2d12 IgG molecules have been found in rabbits of genotype $a^3d^{11}/a^3d^{11}$ (8). κ light chain genes, as well as $V_Ha$ and $C_\gamma d$ heavy chain genes, have been found to encode such unexpected or latent allotypes (4, 9). The existence of these latent allotypes suggests that individual rabbits may carry, in addition to the expected allelic genes that encode the nominal allotypic specificities, one or more genes encoding the latent allotypic specificities. These latent allotype genes are presumably pseudoallelic to those genes encoding the nominal allotypes.

Recent advances in recombinant DNA technology allow direct analysis of the organization of rabbit Ig genes. To this end, a rabbit $C_\kappa$ cDNA and $C_\gamma$ cDNA have been isolated by Heidmann *et al.* (10, 11). To investigate the structure and arrangement of rabbit heavy chain genes, we have constructed a cDNA library using as template RNA from the spleen of a hyperimmunized rabbit and have isolated from this library a plasmid clone, p2a2, that encodes a portion of the $C_\gamma$ gene. The p2a2 cDNA was used as a probe to isolate recombinant phage clones carrying sequences homologous to p2a2 from a library of rabbit liver DNA. We report here the characterization of two different genomic clones.

## MATERIALS AND METHODS

**Construction of cDNA Library.** A rabbit hyperimmunized with *Micrococcus lysodeikticus* was provided by L. S. Rodkey. This rabbit, 79-5, appeared to be homozygous $a^3n^{81}de^{11,15}f^{72}g^{74}$, as determined serologically by A. Gilman-Sachs (*n*), W. J. Mandy (*de*), and W. C. Hanly (*f,g*). RNA was isolated by homogenization of this rabbit's spleen in buffer containing 4 M guanidinium thiocyanate/1 M 2-mercaptoethanol (12, 13) followed by equilibrium centrifugation of the homogenate in CsCl. The poly(A)$^+$RNA was selected by oligo(dT)-cellulose chromatography, and double-stranded cDNA was synthesized essentially as described (14) using oligo(dT) as primer. The *Escherichia coli* polymerase I was a gift from M. Bond, and RNA-dependent DNA nucleotidyltransferase (reverse transcriptase) was obtained from J. Beard (Life Sciences, St. Petersburg, FL). Double-stranded cDNA was passed over a Bio-Gel A-150m column (Bio-Rad) to select the largest molecules (average size, 600 nucleotides). Terminal deoxynucleotidyltransferase (P-L Biochemicals) was then used to add 15–30 dCMP molecules to each 3′ end (15). The dCMP-tailed cDNA was hybridized to *Pst* I-digested, dGMP-tailed pBR322 as described (16). The plasmids were then used to transform (16, 17) *E. coli* MC1061 cells, originally provided by M. Casadaban and obtained from T. Sargent.

**Selection of $C_\gamma$ cDNA.** Tetracycline-resistant bacterial colonies were grown on nitrocellulose filters as described by Hanahan and Meselson (18). Several colonies were picked and tested for the presence of $C_\gamma$ sequences by hybridization with a $^{32}$P-labeled 4.8-kilobase (kb) *Xba* I fragment of mouse genomic DNA encoding $C_\gamma 3$ (19). Of those colonies that hybridized with the mouse $C_\gamma 3$ probe, one, p2a2, was selected for further study.

**DNA Sequence Analysis.** Restriction endonuclease digests of p2a2 or purified fragments were end labeled by using crude aqueous [γ-$^{32}$P]ATP (ICN) and polynucleotide kinase (P-L Biochemicals) as described (20). Nucleotide sequence was determined by the method of Maxam and Gilbert (21).

**Hybridization Analysis of Rabbit Genomic DNA.** High molecular weight DNA was isolated as described (22) from sperm cells of a rabbit, 79-17 (Ig allotypes, a2, a3; d11, d12, e15; f69g77, f72g74; b4; c7). Ten micrograms of DNA was digested with *Eco*RI or *Bam*HI, electrophoresed in an agarose gel, transferred to nitrocellulose (23), and hybridized with $^{32}$P-labeled p2a2 as described (13). After hybridization, the nitrocellulose

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Abbreviations: V and C, variable and constant regions, respectively, of the Ig chain; kb, kilobase(s).

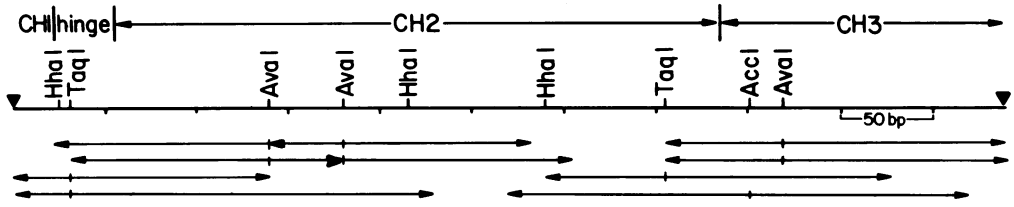


FIG. 1. Restriction map and strategy for nucleotide sequence analysis of p2a2 cDNA. ▼, Positions of the G-C tails added during construction of the library; arrows below the map, end-labeled restriction sites and direction of sequence analysis.

was exposed to preflashed Kodak XAR-5 film for 10 days with an enhancing screen.

**Screening the Rabbit Genomic Library.** A library of rabbit liver DNA in phage Charon 4A was provided by T. Maniatis (24). Approximately $2 \times 10^6$ recombinant phage (2.5 genome equivalents) were plated on *E. coli* LE392 ($4 \times 10^4$ plaque-forming units per 150-mm Petri plate). The library was screened as described (24, 25) using as a probe p2a2 labeled with $^{32}P$ to $1 \times 10^8$ cpm/$\mu$g by nick-translation. Phage that contained sequences homologous to p2a2 were subsequently plaque purified, and DNA was prepared as described by Maniatis *et al.* (24).

**Subclones of Phage DNA.** Phage DNAs were subcloned into plasmid vectors as follows. DNA from phage clone 39-1a was digested with *Eco*RI/*Hin*dIII and electrophoresed in an agarose gel. The 3.4-kb fragment was isolated by electroelution and ligated into plasmid pBR322 digested with *Eco*RI/*Hin*dIII. Several ampicillin-resistant colonies were tested for the presence of a 3.4-kb insert by restriction analysis of isolated plasmids. DNA from phage clone 20b was digested with *Eco*RI and ligated directly into the *Eco*RI site of plasmid pACYC184. Tetracycline-resistant chloramphenicol-sensitive colonies were tested by restriction analysis of isolated plasmids. Clones containing each of the three *Eco*RI fragments of the recombinant insert of phage clone 20b were isolated.

## RESULTS

**Identification and Characterization of a $C_\gamma$ cDNA Clone.** Plasmid DNA from bacterial clones selected from the cDNA library was digested with *Pst* I and analyzed by agarose gel electrophoresis and Southern blot hybridization. One clone, p2a2, contained a 570-base pair insert that hybridized with cloned mouse $C_\gamma$ DNA. To ascertain that this cDNA encoded the rabbit $C_\gamma$ heavy chain, the nucleotide sequence of p2a2 was determined. A restriction map and the strategy for sequence analysis are shown in Fig. 1, and the DNA sequence for the entire insert (538 nucleotides excluding dCMP tails) is presented in Fig. 2. The DNA sequence was translated into amino acid sequences in all reading frames, and one corresponded to amino acids 208–390 (Eu numbering) of rabbit $\gamma$ heavy chains (26, 27).

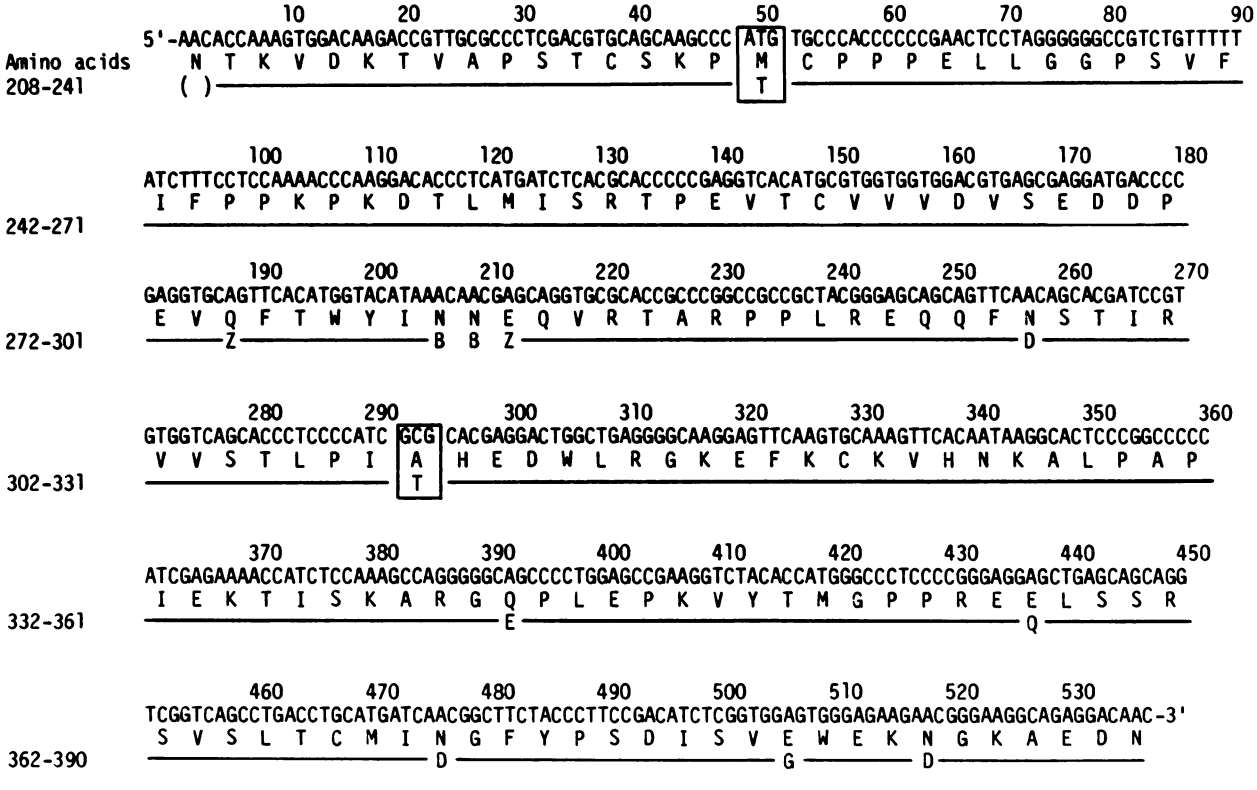


FIG. 2. Nucleotide sequence of p2a2 cDNA. The amino acid translation of the nucleotide sequence is shown on the second line, and the published amino acid sequence (26, 27) of residues 208–390 (Eu numbering system) of the heavy chain of IgG from pooled normal sera is shown on the third line. The solid line indicates that the same residue was found by amino acid sequence analysis as was predicted from the nucleotide sequence of p2a2. Nucleotides 1–24 encode the COOH-terminal end of the CH1 domain (amino acids 208–215), nucleotides 25–57 encode the hinge region, nucleotides 58–390 encode the CH2 domain, and nucleotides 391–538 encode the $NH_2$-terminal half of the CH3 domain. Boxed residues, amino acid positions 224 and 309, indicate the amino acid correlates of the $C_\gamma$d and $C_\gamma$e allotypic specificities, respectively.

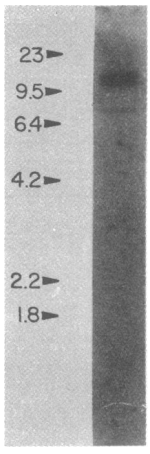


FIG. 3. Hybridization of rabbit 79-17 sperm DNA to $^{32}$P-labeled p2a2 $C_\gamma$ cDNA. Hybridization was at 68°C for 24 hr in 0.45 M NaCl/0.045 M Na citrate/0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin (10× Denhardt's solution)/0.1% NaDodSO$_4$ containing salmon sperm DNA at 50 μg/ml, and poly(rA) at 10 μg/ml. The filter was washed in 0.15 M NaCl/0.015 M Na citrate/0.1% NaDodSO$_4$/0.1% Na pyrophosphate. Specific activity of the $^{32}$P-labeled p2a2 was $1.4 \times 10^8$ cpm/μg. Sizes were estimated from a *Hin*dIII digestion of bacteriophage λ DNA.

Thus, the cDNA encodes the COOH-terminal eight amino acids of the CH1 domain, the hinge region, the CH2 domain, and the $NH_2$-terminal half of the CH3 domain. No 3′-untranslated sequences are present.

Of the 179 amino acid residues encoded by the p2a2 cDNA, seven differences were found between the published amino acid sequence and that derived from the nucleotide sequence (Fig. 2). Residue 380 is glutamic acid rather than glycine as determined by amino acid sequence analysis; the other six differences are aspartic acid/asparagine or glutamic acid/glutamine interchanges. In addition, amino acid 208, which was not identified in the published amino acid sequence, is shown to be asparagine. The four other amino acid interchanges shown in Fig. 2 represent clarification of residues previously reported as aspartic acid/asparagine or glutamic acid/glutamine.

The $C_\gamma$ allotypes of the rabbit from which the cDNA library was derived were d11,e15. Studies of IgG proteins have shown that the d11/d12 allotypic specificities correlate with a methionine/threonine interchange at residue 224 (28) and that the e14/e15 allotypic specificities correlate with a threonine/alanine interchange at residue 309 (29). The nucleotide sequence of p2a2 encodes methionine at position 224 (d11) and alanine at position 309 (e15) (boxed residues in Fig. 2), consistent with the d11,e15 phenotype.

**Hybridization Analysis of Rabbit Genomic DNA.** *Eco*RI-digested rabbit sperm DNA was hybridized with $^{32}$P-labeled p2a2 by using the Southern blot technique. Two major DNA fragments, 10.2 kb and 9.7 kb, and a less intense band of 7.3 kb, were detected (Fig. 3). Comparable results were obtained with *Bam*HI-digested DNA in that two fragments of 16–18 kb hybridized strongly to p2a2. The results of this experiment suggest, but do not prove, that at least two different $C_\gamma$ genes are present in the rabbit haploid genome.

**Identification of Genomic Clones Hybridizing with p2a2 cDNA.** Initial screening of a genomic library of rabbit liver DNA with $^{32}$P-labeled p2a2 resulted in isolation of nine clones. Comparison of the patterns of restriction fragments of the DNA molecules showed that two different DNA segments were represented in these nine clones. The restriction map of clone 39-1a, containing 15.8 kb of rabbit DNA (Fig. 4), was representative of seven of the nine clones. Of these seven, five (including 39-1a) were identical; the other two were identical except for a 1.2-kb deletion in the internal 4.5-kb *Eco*RI/*Hin*dIII fragment. The deletion appeared to be the same in both clones and thus was apparently introduced during the cloning procedure. The restriction maps for the remaining two clones, 20b (containing 12.8 kb of rabbit DNA) and 13-2b (containing 16.4 kb of rabbit DNA), show that the DNA segments contained in these phage clones were derived from overlapping regions of the rabbit genome (Fig. 3). These two overlapping clones have a total of 20.4 kb of contiguous DNA. Southern blot hybridization analysis showed that sequences homologous to p2a2 are found in *Eco*RI fragments of 8.4 kb (clone 39-1a), 2.3 kb (clone 20b), and 2.0 kb (clone 13-2b). Because the library was constructed by adding *Eco*RI linkers to rabbit DNA that had been partially digested with *Hae* III and *Alu* I (24), the terminal *Eco*RI sites in these phage clones are not authentic sites found in the rabbit genome, and thus the 2.0-kb *Eco*RI fragment of clone 13-2b and the 8.4-kb *Eco*RI fragment of clone 39-1a are artificially truncated compared with actual DNA fragments expected in the genomic DNA. The restriction map of clone 39-1a shows no similarity in any orientation to the overlapping maps of clones 20b and 13-2b, and thus the two DNA segments represented by these three phage clones appear to have been derived from distinct regions of the rabbit genome.

**Subclones and Detailed Restriction Maps of Phage Clones.** To study the genomic clones in greater detail, plasmid subclones containing the restriction fragments that hybridized with the $C_\gamma$ cDNA were prepared. These subclones were mapped with nine restriction enzymes. Fig. 5 shows that several restriction site differences occur both 5′ and 3′ to the region that hy-

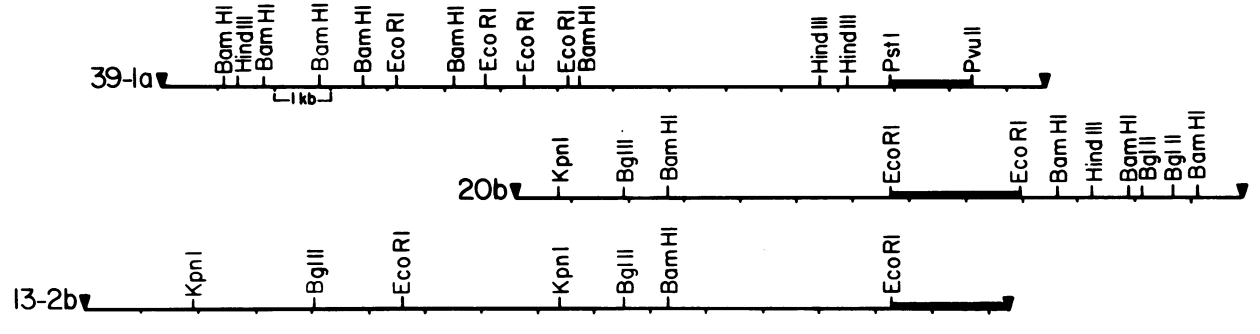


FIG. 4. Restriction maps of three phage clones isolated from a library of rabbit liver DNA. Recombinant inserts are shown such that the 10.7-kb arm of the Charon 4A vector is to the left and the 19.2-kb arm is to the right. Heavy lines indicate restriction fragments that hybridize with p2a2 $C_\gamma$ cDNA. In clone 39-1a, the positions of the *Pst* I and *Pvu* II sites were determined from a subclone of the 3.4-kb *Eco*RI/*Hin*dIII fragment; other *Pst* I and *Pvu* II sites are not shown. ▼, Synthetic *Eco*RI linker sites added during construction of the library.

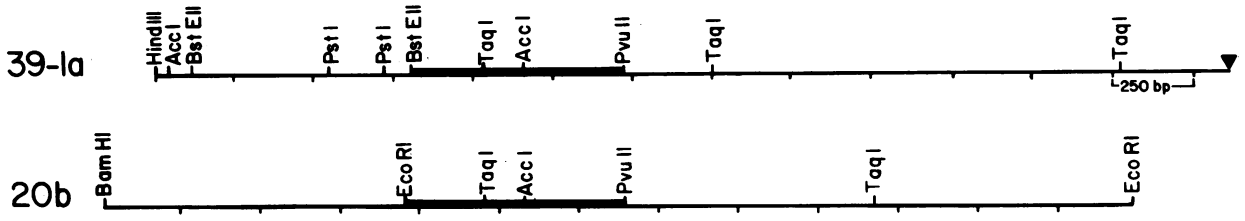


FIG. 5. Restriction maps of subclones of phage 39-1a and 20b. Heavy lines indicate restriction fragments that hybridize with p2a2 $C_\gamma$ cDNA. The subclones are drawn in 5'-3' orientation based on preliminary nucleotide sequence analysis. ▼, Position of the *Eco*RI linker added during construction of the library.

bridizes with the CH2 and CH3 domain coding sequences of the $C_\gamma$ cDNA. In the hybridizing region, some sites appear to be colinear. The considerable differences in these restriction maps are consistent with the two clones having been derived from different regions of the genome.

## DISCUSSION

A cDNA that encodes a portion of the constant region of a rabbit IgG heavy chain ($C_\gamma$) has been synthesized using as template total poly(A)$^+$RNA from the spleen of a hyperimmune rabbit. The nucleotide sequence of the cDNA shows that it encodes 179 amino acids of the $C_\gamma$ chain. This cDNA was used to screen a recombinant phage library of rabbit DNA to isolate clones that contain $C_\gamma$ sequences. Clones were isolated that represent two distinct DNA segments, suggesting that the rabbit genome may contain at least two different genes for $C_\gamma$.

The cDNA described here, p2a2, was isolated from a cDNA library synthesized from the splenic RNA from a normal hyperimmunized rabbit. As expected, the amino acid sequence, translated from the nucleotide sequence, was essentially identical to the published amino acid sequence of rabbit IgG heavy chains obtained from pooled normal sera (26, 27). The only significant difference is at residue 380, which was shown to be glutamic acid by nucleotide sequence analysis. From amino acid sequence analysis of pooled normal IgG, this residue was reported to be glycine (26); however, D. Klapper and T. J. Kindt (personal communication) have recently determined that residue 380 was glutamic acid in heavy chains of a preparation of antibody of restricted heterogeneity. Therefore, either residue 380 is in fact glutamic acid or the glycine/glutamic acid difference may reflect a genetic variation of rabbit IgG that has not been identified in pooled normal IgG. Sequence analysis of other $C_\gamma$ cDNA clones from rabbits of different genetic backgrounds will clarify this situation.

Unlike other species—e.g., humans and mice—the rabbit has only a single subclass of IgG that has been well characterized and thus only one $C_\gamma$ gene per haploid genome was expected. The phage clone 39-1a, isolated from a library of rabbit liver DNA, showed little similarity in its restriction map to maps of phage clones 13-2b and 20b. Thus, it appears that clone 39-1a may have been derived from a different part of the genome than were 13-2b and 20b, in which case these three clones would encode two different $C_\gamma$ genes. Furthermore, p2a2 hybridized strongly to two fragments of *Eco*RI-digested rabbit sperm DNA, consistent with the suggestion that the rabbit genome contains at least two $C_\gamma$ genes. The third band, which hybridized weakly to p2a2 on the blot, apparently reflects a gene segment that is partially homologous to the $C_\gamma$ cDNA.

The phage clone 39-1a, which contains an *Eco*RI fragment of 8.4 kb, is presumably represented in the 10.2-kb or the 9.7-kb fragment detected in the genomic blot of *Eco*RI-digested sperm DNA. This segment of DNA was apparently cleaved during construction of the phage library to produce the 8.4-kb *Eco*RI fragment. The phage clone 20b contains a 2.3-kb *Eco*RI fragment. A band of this size was not detected on the genomic blot, perhaps because of genetic differences between rabbit 79-17 and the donor rabbit used to construct the phage library. No DNA was available from the donor rabbit, and its Ig allotypes are unknown; thus, no direct comparison can be made between the 20b phage clone and the genomic blot.

The identification of two different genomic clones containing sequences that hybridize with the $C_\gamma$ cDNA suggests that the rabbit genome may contain two different genes encoding $C_\gamma$. One gene presumably encodes the major species of $C_\gamma$ found in rabbit serum. The second gene could encode another normally expressed $C_\gamma$—i.e., a second subclass of IgG. The existence of a second subclass was suggested by Rodkey and Freeman (30), who identified, by immunoelectrophoresis, an electrophoretically distinct population of IgG molecules in rabbit serum. As yet, this protein has not been isolated or further characterized. Alternatively, the second gene could encode a $C_\gamma$ that is under regulatory control—i.e., a latent allotype molecule. Although all family studies indicate that the $C_\gamma$de allotypes are controlled by allelic genes, several investigators (4–9) have described rabbits with Ig molecules bearing allotypes not expected from the animals' pedigrees. If $C_\gamma$de latent allotypes reflect the presence of more than one $C_\gamma$ structural gene per haploid genome, then some $C_\gamma$de structural genes would not be allelic but rather pseudoallelic to each other. The fact that the $C_\gamma$ allotypes appear to be allelically encoded may reflect control of these structural $C_\gamma$ genes by allelic regulatory elements. Such regulatory elements have been postulated (31–33) but no direct supporting evidence for them has been found. Analysis of germ-line DNA from rabbits of known genotype will help elucidate the nature of the genes regulating the expression of nominal and latent allotypes.

It is also possible that the second $C_\gamma$ gene may represent a pseudogene—i.e., a gene that cannot be expressed because of a mutation that has produced a stop codon within the coding sequences, a frame shift, defective RNA splice site, or error in promotor or other control sequences (34, 35). Pseudogenes have been found in studies of several gene families, including $V_H$ (36, 37), $J_H$ (38), and *H-2* (39) genes in mice. Nucleotide sequence analysis is necessary to determine whether both $C_\gamma$ genes described here are functional.

Although it appears that the two clones were derived from different regions of the genome, we cannot rule out the possibility that the two DNA segments represent allelic genes. It is not possible to predict how much similarity there could be between alleles. Alleles of the mouse $\gamma$1 gene differ in sites for only one of nine restriction enzymes tested, while alleles of the mouse $\mu$ gene differ in sites for three of five enzymes (40). Restriction site polymorphisms clearly exist in rabbit $C_\gamma$ genes, since the 2.3-kb *Eco*RI fragment and the 7.1-kb *Bam*HI fragment of clone 20b, which hybridized with the $C_\gamma$ cDNA, were not detected in the blot analysis of sperm DNA. Also, Heidmann and Rougeon (11) showed restriction site polymorphisms

in the *Eco*RI digests of liver DNA from different rabbits, although they did not specify whether the donor rabbits were homozygous or heterozygous at the $C_\gamma d$ and $C_\gamma e$ loci. In addition, they showed a genomic 3.5-kb *Pst* I fragment that hybridized with their $C_\gamma$ cDNA encoding the CH3 domain. This fragment is not present in clone 20b but might correspond to a *Pst* I fragment of clone 39-1a that extends beyond the end of this clone. Thus, restriction site polymorphisms may be common among rabbits of different heavy chain haplotypes. Since the $C_\gamma$ allotypes of the donor rabbit from which the genomic library was made are unknown, the two clones described here could conceivably represent allelic forms of one gene, even though there are multiple restriction site differences between them.

The rabbit liver DNA library was constructed by using the λ phage vector Charon 4A (41) and was amplified after initial plating. Thus, it is possible that $C_\gamma$ clones other than those isolated in this study existed but were lost during amplification. An artifact of amplification is the most likely explanation for finding several clones identical to clone 39-1a in the initial library screening.

Further analysis of the two $C_\gamma$ genomic clones described here will allow us to establish the relationships between them and to determine the nature and location of control sequences in the flanking regions of the $C_\gamma$ genes. These clones and the p2a2 cDNA will be useful in studies of the control of expression of the complex rabbit Ig heavy chain gene cluster.

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